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Analysis of the σ^E Regulon in Crohn's Disease-Associated *Escherichia coli* Revealed Involvement of the *waaWVL* Operon in Biofilm Formation

Benoit Chassaing,[a,b*] Estelle Garénaux,[a,b] Jessica Carriere,[a,b] Nathalie Rolhion,[a,b*] Yann Guérardel,[c,d] Nicolas Barnich,[a,b] Richard Bonnet,[a,b,e] Arlette Darfeuille-Michaud[a,b,e]

Clermont Université, UMR 1071 Inserm, Université Auvergne, Clermont-Ferrand, France[a]; INRA USC 2018, Clermont-Ferrand, France[b]; Université de Lille 1, Unité de Glycobiologie Structurale et Fonctionnelle, Villeneuve d'Ascq, France[c]; CNRS, UMR 8576, Villeneuve d'Ascq, France[d]; Centre Hospitalier Universitaire, Clermont-Ferrand, France[e]

ABSTRACT

Ileal lesions of patients with Crohn's disease are colonized by adherent-invasive *Escherichia coli* (AIEC), which is able to adhere to and to invade intestinal epithelial cells (IEC), to replicate within macrophages, and to form biofilms on the surface of the intestinal mucosa. Previous analyses indicated the involvement of the σ^E pathway in AIEC-IEC interaction, as well as in biofilm formation, with σ^E pathway inhibition leading to an impaired ability of AIEC to colonize the intestinal mucosa and to form biofilms. The aim of this study was to characterize the σ^E regulon of AIEC strain LF82 in order to identify members involved in AIEC phenotypes. Using comparative *in silico* analysis of the σ^E regulon, we identified the *waaWVL* operon as a new member of the σ^E regulon in reference AIEC strain LF82. We determined that the *waaWVL* operon is involved in AIEC lipopolysaccharide structure and composition, and the *waaWVL* operon was found to be essential for AIEC strains to produce biofilm and to colonize the intestinal mucosa.

IMPORTANCE

An increased prevalence of adherent-invasive *Escherichia coli* (AIEC) bacteria was previously observed in the intestinal mucosa of Crohn's disease (CD) patients, and clinical observations revealed bacterial biofilms associated with the mucosa of CD patients. Here, analysis of the σ^E regulon in AIEC and commensal *E. coli* identified 12 genes controlled by σ^E only in AIEC. Among them, WaaWVL factors were found to play an essential role in biofilm formation and mucosal colonization by AIEC. In addition to identifying molecular tools that revealed a pathogenic population of *E. coli* colonizing the mucosa of CD patients, these results indicate that targeting the *waaWVL* operon could be a potent therapeutic strategy to interfere with the ability of AIEC to form biofilms and to colonize the gut mucosa.

Crohn's disease (CD) and ulcerative colitis (UC) are multifactorial diseases that occur in individuals with genetic predispositions and in whom an environmental or infectious trigger causes an abnormal immune response (1, 2). Clinical observations show that bacterial biofilms are associated with the mucosa of inflammatory bowel disease (IBD) patients (3). The mean density of the mucosal biofilm is 2-fold higher in IBD patients than in patients with inflammatory bowel syndrome or controls, and the bacteria are mostly adherent (3). Other lines of evidence suggest that bacteria play a role in the onset and perpetuation of IBD (4, 5). Several independent studies have reported the abnormal presence of adherent-invasive *E. coli* (AIEC) bacteria in the ileal mucosa of CD patients (6–12). In addition to their ability to adhere, these *E. coli* bacteria are able to invade intestinal epithelial cells (IEC). The adhesion and invasion process of the reference AIEC strain LF82 involves type 1 pili, flagella, outer membrane proteins, outer membrane vesicles, and long polar fimbriae (13–17). In addition, analysis of the genome sequence of AIEC strain LF82 revealed the presence of several known virulence genes and four putative pathogenic islands carrying virulence-related genes (18).

We previously reported that the σ^E pathway plays a crucial role in AIEC strain LF82 but not in nonpathogenic *E. coli* K-12 MG1655 by regulating adhesion, invasion, and biofilm formation processes (19). However, the molecular link between σ^E pathway activation and AIEC phenotypes is still unclear. The σ^E factor, also called RpoE, is activated by stresses that interfere with the folding of outer membrane proteins (OMPs) (20–22), such as the osmolarity encountered in the gastrointestinal tract (15). As expected

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Address correspondence to Benoit Chassaing, chassaingbenoit@yahoo.fr, or Richard Bonnet, richard.bonnet@udamail.fr.

* Present address: Benoit Chassaing, Institute for Biomedical Sciences, Center for Inflammation, Immunity and Infection, Georgia State University, Atlanta, Georgia, USA; Nathalie Rolhion, Institut Pasteur, Unité des Interactions Bactéries-Cellules, Paris, France.

E.G. and J.C. contributed equally to this work.

This article is dedicated to our esteemed mentor and coauthor Arlette Darfeuille-Michaud, who sadly passed away on 28 June 2014 (68).

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from its role in the stress response, the σ^E regulon includes genes that encode periplasmic foldases, proteases, and chaperones that aid in OMP folding. In addition, σ^E transcribes an array of biosynthetic enzymes involved in phospholipid, fatty acid, lipopolysaccharide (LPS), and membrane-derived oligosaccharide synthesis and transport, as well as a number of other cell envelope proteins, including lipoproteins, inner membrane proteins, and envelope proteins of unknown function (23–25).

The aim of the present study was to decipher the molecular mechanism of σ^E pathway involvement in the pathogenesis of AIEC and to identify AIEC virulence factors with σ^E-regulated expression. We report here the involvement of the σ^E-mediated pathway in the ability of AIEC strains to form biofilms and to colonize the intestinal mucosa via transcription of the *waaWVL* operon. This operon is transcribed in response to σ^E pathway activation, is involved in AIEC lipopolysaccharide synthesis, and is essential for AIEC strains to produce biofilm and to colonize the intestinal mucosa.

MATERIALS AND METHODS

Ethics statement. Animal protocols were approved by the Committee for Ethical Issues, CEMEA Auvergne (permit number CE16-0927-2956), and all animals were used in accordance with the European Community guidelines for the care and use of animals (86/609/CEE).

Reference bacterial strains, plasmids, and cell lines. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Bacteria were grown routinely in LB broth (BD) overnight at 37°C without shaking. Antibiotics were added to medium at the following concentrations: ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and chloramphenicol (25 μg ml^{-1}).

When experiments involved RseA/B and RpoE protein overexpression, a concentration of 20 g $liter^{-1}$ of arabinose was used; we previously reported that this concentration leads to a strong and reliable decrease in σ^E pathway activity (19). For experiments involving induction of *waaWVL* expression (see Fig. 3B and 7; see Fig. S1 and S4 in the supplemental material), a concentration of 5 g $liter^{-1}$ of arabinose was used.

Intestine 407 cells (I-407; derived from human intestinal embryonic jejunum and ileum) were purchased from Flow Laboratories, Inc., McLean, VA. Cultured cells were maintained in an atmosphere containing 5% CO_2 at 37°C in modified Eagle medium (Seromed; Biochrom KG, Berlin, Germany) supplemented with 10% (vol/vol) fetal bovine serum (Lonza), 1% nonessential amino acids (Lonza), 1% L-glutamine (Lonza), 200 U of penicillin, 50 μg of streptomycin, and 0.25 μg of amphotericin B per liter, and with 1% minimal essential medium (MEM) vitamin mix X-100 (Lonza).

Construction of isogenic mutants and *trans*-complementation assays. Isogenic mutants were generated with a PCR product using the method described by Datsenko et al. (26) and modified by Chaveroche et al. (27). Primers used are listed in Table S2 of the supplemental material. For *trans*-complementation assays, a PCR product containing the entire 3,389-bp *waaWVL* operon was cloned into the pBAD24 vector (28), and the *rseAB* operon was cloned into the pBAD33 vector, as previously described (19) (see Tables S1 and S2 in the supplemental material).

σ^E-binding consensus sequence elaboration and determination of σ^E-regulated genes in AIEC reference strain LF82 and *E. coli* K-12 strain MG1655. The HMMER software program (version 3.0; http://hmmer.janelia.org/) was used for the identification of the σ^E-binding motifs within 28 sequences previously reported to be bound by the σ^E protein (23) (see Table S3 in the supplemental material). HMMER uses probabilistic models called profile hidden Markov models to identify likely motifs within the input set of sequences. It produces a consensus sequence and a position-specific probability matrix, which provides probabilities associated with each base at each position. The aligned σ^E promoter sequences were visualized using sequence logo (29) (http://weblogo.berkeley.edu/). We then applied the HMMER program, using the motif matrix previously determined, to search for the motif in the whole genome of *E. coli* K-12 strain MG1655 and AIEC reference strain LF82 (18, 30). The algorithm in HMMER calculates position *z*-scores for the motif at each possible position within a sequence. Only the motif hits with a HMMER score higher than 1 and located less than 1,100 bp from the translation start point were considered putative σ^E-binding sites (23).

Promoter expression assay. To generate the *lacZ* fusion promoter, promoters of genes *topA*, ORF1, *ychH*, and of operon *waaWVL* were amplified by PCR (see Table S2 in the supplemental material). The resulting 360-bp fragments contained the promoter sequence of the corresponding gene/operon as well as the putative RpoE-binding site identified by *in silico* analysis. These PCR fragments were then ligated into the plasmid vector pRS550 (31) and designated pRS550-*topA*, pRS550-ORF1, pRS550-*waaW*, and pRS550-*ychH*, respectively. β-Galactosidase activities were analyzed with a β-galactosidase assay kit (Qiagen) with strains harboring these pRS550 constructs as well as the pBAD30 empty vector or pBAD30-*rpoE* in LB culture medium. β-Galactosidase activity of each sample was determined by measuring the optical density at 420 nm (OD_{420}) at 24 h, and the number of bacteria in each sample was calculated based on OD_{620} measurements, from which Miller units were determined.

RNA manipulations, reverse transcription (RT), and RT-PCR. Cultures were grown at 37°C in LB, LB plus 20 g $liter^{-1}$ NaCl, cell culture medium (DMEM plus 10% fetal bovine serum) containing 2% sodium choleate, M9 minimal medium (Invitrogen) supplemented with glucose at 4 g $liter^{-1}$, $CaCl_2$ at 0.1 mM, and $MgSO_4$ at 2 mM, or M63 minimal medium (U.S. Biological) supplemented with glucose at 8 g $liter^{-1}$ and $MgSO_4$ at 1 mM. At an OD_{620} of 0.2 and when needed, L-arabinose at 20 g $liter^{-1}$ was added to induce the overexpression of RseAB, and L-arabinose at 5 g $liter^{-1}$ was added to induce the overexpression of WaaWVL. Total RNA was extracted at 4 h, 16 h, 24 h, or from overnight-cultured bacteria and treated with DNase (Roche Diagnostics) to remove contaminating genomic DNA.

For RNA extraction of biofilm-associated bacteria, strains were grown overnight in Luria-Bertani broth with 5 g $liter^{-1}$ of glucose (Euromedex) at 35.5°C, after which 1/100 dilutions were made in M63 minimal medium (U.S. Biological) supplemented with 8 g $liter^{-1}$ glucose. Fifteen-milliliter aliquots were then placed in wells of non-cell-treated polystyrene petri plates and incubated at 30°C without shaking. At different time points, plates were washed once, bacteria were harvested using a scraper, and RNAs were extracted as previously described. Bacterial growth in M63 minimal medium supplemented with 8 g $liter^{-1}$ (0.8%) glucose was used as a control.

The RNAs were reverse transcribed and amplified using primers specific to *rpoE*, *yjiW*, *lpfA*, ORF1, ORF2, *yliF*, *ygcU*, *waaWVL*, ORF3, *gnd*, *uidC*, *yafT*, and *ychH* mRNAs or 16S rRNA (see Table S2 in the supplemental material). Amplification of a single expected PCR product was confirmed by electrophoresis on a 2% agarose gel. RT-PCR was performed using an Eppendorf Realplex system, and the RNA levels were quantified using RNA master SYBR green I (Roche Diagnostic) with 0.25 μg of total RNA.

Adhesion and invasion assay. The bacterial adhesion assay was performed as described previously (32). Briefly, intestine 407 cells were seeded in 24-well tissue culture plates with 4×10^5 cells per well. Monolayers were then infected at a multiplicity of infection of 10 bacteria per cell in 1 ml of cell culture medium without antibiotics and with heat-inactivated fetal calf serum (FCS; PAA Laboratories). After a 3-h incubation period at 37°C, monolayers were washed three times in phosphate-buffered saline (PBS, pH 7.2). Epithelial cells were then lysed with 1% Triton X-100 (Euromedex) in deionized water. Samples were diluted and plated onto Mueller-Hinton agar plates to determine the number of CFU corresponding to the total number of cell-associated bacteria (adherent and intracellular bacteria). To determine the number of intracellular bacteria, fresh cell culture medium containing 100 μg ml^{-1} gentamicin was

added for 1 h to kill extracellular bacteria. Monolayers were then lysed with 1% Triton X-100, and bacteria were quantified as described above.

Biofilm formation assays. Biofilm formation assays were performed using a previously described method (33). Strains were grown overnight in Luria-Bertani broth with 5 g liter^{-1} of glucose (Euromedex) at 35.5°C, after which 1/100 dilutions were made in M63 minimal medium (U.S. Biological) supplemented with 8 g liter^{-1} (0.8%) glucose. Aliquots (130 μl) were then placed in wells of non-cell-treated polystyrene microtiter plates and incubated overnight at 30°C without shaking. Afterwards, ODs were read at 630 nm in order to determine bacterial growth. Wells were washed once, adherent bacteria were stained with 1% crystal violet solubilized in ethanol, and ODs were read at 570 nm. Specific biofilm formation (SBF) was calculated using the following formula: SBF = (AB − CW)/G, in which AB is the OD_{570} of the attached and stained bacteria, CW is the OD_{570} of the stained control wells containing only bacterium-free medium (to eliminate unspecific or abiotic OD values), and G is the OD_{630} as a measure of cell growth in broth (34, 35). Assays were performed in triplicate.

Biofilm formation assays were also performed using paraformaldehyde (PFA)-fixed intestinal epithelial I-407 cell monolayers. Briefly, confluent I-407 monolayers were fixed for 15 min in 4% PFA. After washing, bacterial strains expressing green fluorescent protein (GFP) (36) and diluted in M63 medium supplemented with 8 g liter^{-1} glucose were applied and incubated overnight at 30°C without shaking. For visualization, infected epithelial monolayers were fixed again for 15 min in 4% PFA, phalloidin-tetramethyl rhodamine isothiocyanate (TRITC) was used to visualize actin, and Hoechst stain was used to visualize nuclei. The slides were examined with a Zeiss LSM 510 Meta confocal microscope.

Image processing via COMSTAT. Images of biofilms at the surface of intestinal epithelial cell I-407 monolayers were analyzed with the computer program COMSTAT1 (37). A fixed threshold value was used for all image stacks, and values of roughness and thickness were determined.

Mouse ileal loop experiments. Six-week-old FVB wild-type male mice were starved for 24 h before surgery, with water available *ad libitum*. They were anesthetized, and their intestines were exteriorized through a midline incision (38). Two or three intestinal segments (about 1 cm) without Peyer's patches were ligated and inoculated by mixed inocula comprising equivalent numbers (5×10^7 CFU) of bacteria of two strains. Six hours postinfection, the number of each bacterial strain associated with the mucosa of ligated loops was determined to establish the competitive index (CI), which provides a sensitive measurement of the relative degree of attenuation (39). Surgery was performed under ketamine-xylazine anesthesia, and all efforts were made to minimize suffering. Mice were killed by cervical dislocation according to animal care procedures.

LPS purification. AIEC strain LF82 (serotyped as O83:H1) and the LF82-Δ*waaW*::pBAD24-*waaWVL* mutant were grown overnight at 37°C in 150 ml of Luria-Bertani medium with or without 5 g liter^{-1} arabinose. LPS was isolated according to the hot phenol-water procedure (40) with some modifications. Briefly, bacteria were collected by centrifugation, washed twice in PBS containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and then disrupted by sonication. To eliminate remaining nucleic acids and proteins, lysates were treated with 200 μg/ml proteinase K (1 h, 65°C, with gentle mixing) and then with 40 μg/ml DNase and 80 μg/ml RNase (37°C, in the presence of 1 μl/ml 20% $MgSO_4$ and 4 μl/ml chloroform overnight with gentle mixing). Finally, an equal volume of hot (68°C) 90% phenol was added to the mixtures, followed by vigorous shaking at 68°C for 15 min. Suspensions were then cooled on ice and centrifuged at 8,500 × *g* for 15 min. Aqueous phases were pooled, and phenol phases were reextracted with 10 ml distilled water at 68°C. Pooled aqueous phases were extensively dialyzed against distilled water at 4°C, and purified LPS product was finally lyophilized.

SDS-PAGE analysis. LPS (25 μg) was separated by SDS-PAGE on a 4% stacking and 15% separating gel and subsequently revealed by either silver staining, periodic acid-Schiff (PAS) staining, or anti-O83 antigen serum immunoblotting. Rabbit antiserum against *E. coli* LPS O83 was generously provided by Lothar Beutin (Department of Biological Safety, Robert Koch Institut, Berlin, Germany).

LPS composition analysis by nuclear magnetic resonance. Prior to nuclear magnetic resonance (NMR) spectroscopic analysis, samples were repeatedly exchanged in ^2H_2O (99.97% purity; Euriso-top, CEA Saclay, France) with intermediate freeze-drying and then dissolved in 500 μl of D_2O (Euriso-top). Chemical shifts were expressed in parts per million downfield from the signal of the methyl groups of acetone. Samples were analyzed in 5-mm tubes, and one-dimensional proton ^1H experiments were recorded on a Bruker spectrometer at 9.4 T. Assignment of spectra was performed using the Topspin 3.0 program (Bruker Biospin) for spectra visualization and overlap.

Statistical analysis. Numerical values were expressed as means with standard errors of the means (SEM). Statistical comparisons were performed using a 2-tailed Student *t* test, unless the variables required a 2-tailed Fisher exact test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Identification of the σ^E regulon in AIEC strain LF82 and *E. coli* K-12 strain MG1655. The σ^E pathway was previously reported to be involved in the pathogenesis of AIEC strain LF82 by regulating adhesion and invasion of intestinal epithelial cells, as well as the biofilm formation process (19). This occurs through regulation of expression of flagella, type 1 pili, and still-uncharacterized factors involved in the interaction of AIEC bacteria with host cells (15, 19). Our aim in the present study was to search in the genome of strain LF82 for still-unknown virulence factors whose expression is dependent on the σ^E pathway. To identify specific genes whose transcription is regulated by σ^E in strains LF82 and MG1655, the HMMER software program (version 3.0; http://hmmer.janelia.org/) was used to define σ^E-binding consensus motifs by using 28 DNA sequences previously reported to be bound by σ^E (*greA*, *yaeT*, *ygiM*, *rpoH*, *fkpA*, *rpoE*, *bacA*, *yggN*, *yfeY*, *clpX*, *yhjJ*, *yfeK*, *ybfG*, *ddg*, *yfgM*, *plsB*, *mdoG*, *yhbG*, *yfjO*, *rseA*, *yeaY*, *htrA*, *sixA*, *dsbC*, *sbmA*, *yieE*, *yraP*, and *yfgC*) (23) (see Materials and Methods and Table S3 in the supplemental material). The σ^E-binding consensus sequence obtained is presented in Fig. 1A and was used to perform genome-wide predictions of σ^E-binding consensus sites within the genomes of AIEC strain LF82 and K-12 strain MG1655. This method allowed the identification of 53 genes whose transcription is putatively regulated by σ^E in strain MG1655 (see Table S4 in the supplemental material) and of 52 genes whose transcription is putatively regulated by σ^E in strain AIEC strain LF82 (see Table S5 in the supplemental material). Importantly, the comparison of these two σ^E regulons identified 40 genes commonly regulated in the two strains, 13 genes specifically regulated in K-12 strain MG1655 (Table 1) and 12 genes specifically regulated in AIEC strain LF82 (Table 2).

In order to confirm the results of this *in silico* analysis, we intended to measure mRNA levels of the 12 AIEC-specific genes in a mutant of AIEC strain LF82 deleted for the σ^E-encoding gene. However, as previously reported, such a mutant is lethal in *Escherichia coli* (41, 42). In order to counteract this methodology problem, we used the LF82 strain transformed with the pBAD24-*rseAB* plasmid, which allows the expression of the anti-sigma factors RseA and RseB under the control of an arabinose-dependent promoter, preventing σ^E interaction with RNA polymerase (19, 43, 44). As a control, quantification of *rpoE* mRNA levels showed that overexpression of RseAB in a growth medium containing 20 g liter^{-1} arabinose led to decreased expression of the *rpoE* gene in

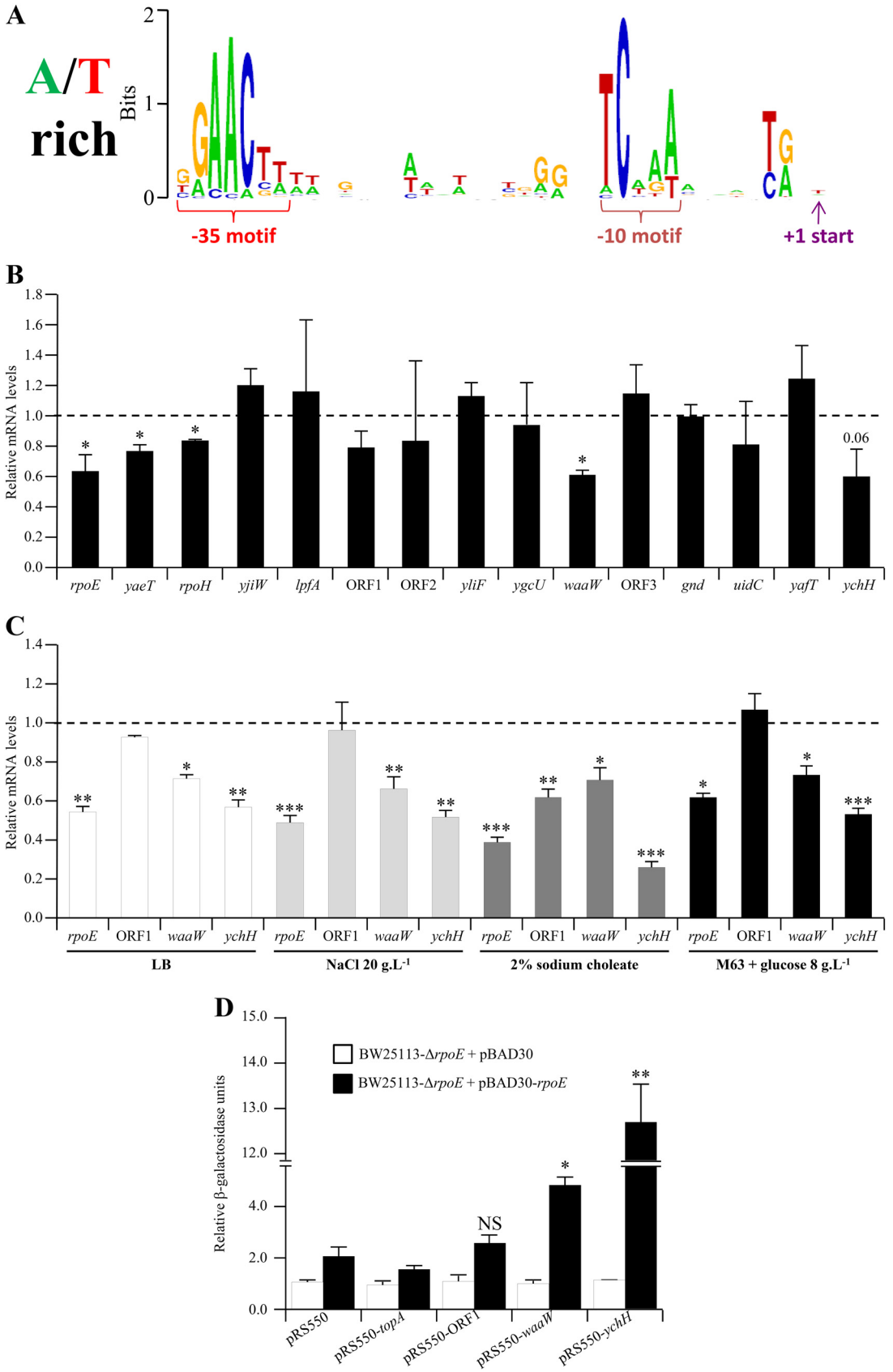
A
A/T
rich
Bits
2
1
0
-35 motif
-10 motif
+1 start
B
Relative mRNA levels
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.06
rpoE
yaeT
rpoH
yjiW
lpfA
ORF1
ORF2
yliF
ygcU
waaW
ORF3
gnd
uidC
yafT
ychH
C
Relative mRNA levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
rpoE
ORF1
waaW
ychH
LB
NaCl 20 g.L-1
2% sodium choleate
M63 + glucose 8 g.L-1
D
Relative β-galactosidase units
15.0
14.0
13.0
12.0
4.0
2.0
0.0
BW25113-ΔrpoE + pBAD30
BW25113-ΔrpoE + pBAD30-rpoE
NS
pRS550
pRS550-topA
pRS550-ORF1
pRS550-waaW
pRS550-ychH

TABLE 1 Predicted RpoE regulon members of commensal *E. coli* K-12 strain MG1655 compared to adherent-invasive *E. coli* strain LF82

Transcription unit	Genome location (bp)	Presence in AIEC strain LF82	HMMER score	Protein function	RpoE promoter sequence[a]
yfjO	2764666/2764732	−	5.46	Hypothetical protein from prophage CP4-57	**CCTGAACT**ACGCACCATTGAAGGTG**TCTTA**AAAAGTAA
ybfG	715860/715926	−	4.73	Hypothetical protein	**AAGGAACT**TAATATTTAAAAAATGT**TCCAT**ACAATTCC
bacA	3202193/3202259	+	4.44	Phosphatase	**GTTAAAC**CAAACGGTTATAACCTGG**TCATA**CGCAGTAG
insH	3651409/3651474	−	3.96	Transposase	**GTATGAA**AGATTGGTTATCCTGGCC**TCTAA**AAATTTAA
araE	2980372/2980437	+	3.69	Arabinose transporter	**TAATGAA**CTTTATGAATTTTATCT**GCTGT**AAAATTAGG
ygcJ	2879889/2879955	−	3.22	Hypothetical protein	**TGGTGAAC**GTTTTGACCAAAAAATCA**TCGAT**AAGACAT
yraH	3285300/3285365	−	2.42	Hypothetical adhesin	**ATCTTAA**AGTTCAGTCTATTTAATGT**TCAAT**GAAATAT
matA	310641/310706	+	1.34	Hypothetical regulatory protein	**ATCGAAA**ATAATTAAACTTAATC**TCGTT**TAACCTTTAT
livK	3595759/3595824	+	1.30	Periplasmic protein	**AAAGGCA**CTTTTTTCTGTTTATCTA**TCAAT**AAATTCAG
rutR	1073295/1073360	+	1.14	Transcriptional regulator	**ATCTAAA**ATATCTGGTAAAAAGTGG**ACTAA**ACGGTCAA
puuA	1359058/1359123	−	1.12	Protein involved in synthesis of gamma-Glu-putrescine	**AAATGAC**CTTTATGTTCAATATTTTTT**TCAAT**CTAGCAG
yfeN	2522140/2522206	+	1.12	Outer membrane protein	**CTTAAAC**CTTCCGCCATATTGGTAA**TCGCA**GAGACCGC
ydiY	1804272/1804336	+	1.04	Hypothetical membrane protein	**TATTTAA**AATTTTGCAGATAAATAT**ATATA**AATAAAAA

[a] Boldface portions of sequences represent −35 and −10 motifs of the putative RpoE-binding site.

the LF82::pBAD24-*rseAB* construct (0.63-fold ± 0.12-fold decrease; $P = 0.045$) (Fig. 1B). In addition, overexpression of RseAB also led to decreased expression of the genes *yaeT* and *rpoH*, whose transcription is under the control of a σ^E-regulated promoter, thereby validating our strategy of utilizing RseAB overexpression (Fig. 1B). Expression analysis of the 12 genes identified *in silico* as having transcription putatively under the control of σ^E in LF82 bacteria but not in MG1655 bacteria (*yjiW*, *lpfABCDE*, ORF1, ORF2, *yliF*, *ygcU*, *waaWVL*, ORF3, *gnd*, *uidC*, *yafT*, and *ychH*) showed that only three of them presented decreased mRNA expression after inhibition of the σ^E pathway: ORF1, specific to LF82 and encoding a hypothetical protein with unknown function; *ychH*, encoding a hypothetical inner membrane protein; and gene *waaW* from the *waaWVL* operon, which encodes three enzymes predicted to be involved in LPS biosynthesis (expression levels relative to the wild-type (WT) strain of 0.78 ± 0.12, 0.61 ± 0.04, and 0.59 ± 0.20, respectively). However, statistical analysis indicated that only the transcription of *waaW* was significantly decreased ($P = 0.039$).

Similar experiments were performed in other culture media, such as LB containing NaCl at 20 g liter^{-1} (previously reported to lead to σ^E pathway activation [15]), cell culture medium containing 2% sodium choleate (previously reported to lead to increased long polar fimbria expression [45]), or M63 medium containing 8 g liter^{-1} glucose (medium used for the biofilm formation assay). Quantification of *rpoE* mRNA levels showed that overexpression of RseAB led to decreased expression of the *rpoE* gene in all the media used (Fig. 1C). Similarly, decreased expression levels of the genes *waaW* and *ychH* were observed under all growth conditions. Of note, with RseAB overexpression, ORF1 expression was decreased only in cell culture medium containing 2% sodium choleate (Fig. 1C).

We next performed a β-galactosidase assay (19, 31) in order to confirm these findings. For this purpose, we cloned DNA sequences encompassing putative ORF1, *ychH*, and *waaWVL* promoters upstream of a *lacZ* reporter gene in the pRS550 plasmid and measured β-galactosidase activity. We found that promoters of these genes led to low β-galactosidase synthesis, and the analysis of decreased β-galactosidase synthesis in response to RseAB overexpression was not viewed as an efficient way to identify regulation of expression of these genes by σ^E. Instead, we decided to measured β-galactosidase activity in the *lacZ*-negative *E. coli* strain BW25113, deleted for the RpoE-encoding gene and complemented with the pBAD30 empty vector or with the pBAD30-*rpoE* vector (46). While this strain is likely to contain suppressor mutations that counteract the lethality normally observed with such a deletion, as previously reported (47), we utilized it as a way of analyzing a potential increase of β-galactosidase activity in the presence versus in the absence of the RpoE protein. Results, presented in Fig. 1D and expressed as the fold variation in strain

FIG 1 (A) Sequence logos of σ^E promoter motifs. Motifs were identified upstream of the 28 mapped transcription starts in *E. coli* K-12 strain MG1655. Sequence logos (http://weblogo.berkeley.edu/) (29) of the −35, −10, and +1 start site motifs and the A/T-rich UP sequences are indicated. (B) mRNA levels of genes belonging to the predicted σ^E-specific regulon of AIEC strain LF82. Results are expressed as the fold variation in the LF82 strain overexpressing the inhibitory complex RseA-RseB of the σ^E pathway, determined using 20 g liter^{-1} arabinose, relative to that of the wild-type strain. 16S rRNA levels were measured as a reference. Data are means ± SEM from three separate experiments. (C) mRNA levels of *rpoE*, ORF1, *waaW*, and *ychH* genes in AIEC strain LF82 grown in LB medium, LB medium plus NaCl 20 g liter^{-1}, cell culture medium plus 2% sodium choleate, or M63 medium plus 8 g liter^{-1} glucose. Results are expressed as the fold variation in the LF82 strain overexpressing the inhibitory complex RseA-RseB of the σ^E pathway, determined by using 20 g liter^{-1} arabinose, relative to that of the wild-type strain. 16S rRNA levels were measured as a reference. Data are means ± SEM from three separate experiments. (D) Activation of *topA*, ORF1, *waaWVL*, and *ychH* promoters in the BW25113 Δ*rpoE* strain transformed with the pBAD30 empty vector or pBAD30-*rpoE* vector in the presence of 20 g liter^{-1} arabinose. Data are presented as β-galactosidase activity of the BW25113 Δ*rpoE*::pBAD30-*rpoE* strain relative to activity of the BW25113 Δ*rpoE*::pBAD30 strain. Data are means ± SEM from three separate experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 2 Predicted RpoE regulon members of adherent-invasive *E. coli* strain LF82 compared to commensal *E. coli* K-12 strain MG1655

Transcription unit	Genome location (bp)	Presence in K-12 strain MG1655	HMMER score	Protein function	RpoE promoter sequence[a]
yjiW	4707553/4707620	+	5.05	Hypothetical endonuclease	**TGAAATT**ATGGATTATTTTATA**ACTCT**AAAGAGTCA
lpfABCDE	3761291/3761358	−	3.55	*Long Polar Fimbrae* encoding operon	**TCAACTT**ATTGCAAAAATTAATAT**TCAGT**AAAAATAA
ORF 1	1494961/1495026	−	3.00	Hypothetical protein, function unknown, with putative DNA-binding homeodomain	**CGAAAAC**TTAAAAAAATAATGCG**TCAGA**TCTGATAAA
ORF 2	2127613/2127679	−	2.40	Hypothetical protein with unknown function, with signal peptide and 5 transmembrane domains	**TTAAAAA**ATTAGTCCCTTCGATTG**TCTCT**ACAGGTGTT
yliF	826377/826442	+	1.74	Hypothetical diguanylate cyclase	**GGAAATT**GGCGAACTATTCCTGG**TCTAT**CAACCGATTG
ygcU	2876616/2876679	+	1.54	Hypothetical dehydrogenase	**GGAAATG**ATTGAAAAACAGGGGG**TCGAA**GTTGAT
waaWVL	3845291/3845359	+	1.53	Involved in LPS biosynthesis	**TGAAATA**CTGGCCTATAATTTTAAA**ACAGT**AAAAGTAT
ORF 3	3121782/3121848	−	1.51	Hypothetical protein with unknown function, with signal peptide and 2 transmembrane domains	
Gnd	2123143/2123209	+	1.30	Gluconate-6-phosphate dehydrogenase	**AGAAACA**TTATCAAAATTAAATTT**ACAAA**AAATATAGG
uidC	1670175/1670241	+	1.24	Hypothetical protein with unknown function, with signal peptide and transmembrane domains	**TCAACGA**TATCACTAGTTAATAT**TCAAT**AAAAATAAT
yafT	241096/241161	+	1.20	Hypothetical aminopeptidase	**TGCACGT**TATGATTTTCATTTT**TCTAT**TGATTTAATG
ychH	1269466/1269531	+	1.15	Hypothetical inner membrane protein	**TGAAATA**AGGGTTGTAATTGTGA**TCACA**CCCGCACATA

[a] Boldface portions of sequences represent −35 and −10 motifs of the putative RpoE-binding site.

BW25113-Δ*rpoE*::pBAD30-*rpoE* compared to strain BW25113-Δ*rpoE*::pBAD30 revealed that expression of RpoE in the presence of 20 g liter^{-1} arabinose led to transcriptional activation of both *waaWVL* and *ychH* promoters. However, the *topA* promoter (a gene that does not harbor any RpoE-binding sequence and was used as a control) and the ORF1 promoter were not activated when RpoE was expressed (Fig. 1D). Altogether, these data confirmed that the *waaWVL* operon and *ychH* gene are new members of the σ^E regulon in the AIEC strain LF82.

Involvement of specific σ^E regulon members in the ability of LF82 to interact with host cells. We next addressed the involvement of the genes *waaWVL* and *ychH*, which belong to the σ^E regulon, in the adhesion and invasion processes of AIEC strain LF82 by generating isogenic mutants. Of note, we failed to obtain any mutant deleted for the *waaW* gene or for the entire *waaWVL* operon, suggesting that deletion of *waaWVL* is lethal in AIEC strain LF82. To overcome this lethality, strain LF82-Δ*waaW*::pBAD24-*waaWVL* was constructed, and the phenotype of the resulting construct was analyzed in the absence of arabinose. This model allowed very low basal expression of all three transcripts, *waaW*, *waaV*, and *waaL*, as a consequence of leaky expression from the pBAD promoter, which can counteract lethality (see Fig. S1 in the supplemental material), and *waaW*, *waaV*, and *waaL* transcript expression levels were fully restored in the presence of 5 g liter^{-1} arabinose (see Fig. S1). Complementation was performed with all three genes, since *waaWVL* is an operon, and the deletion of *waaW* was found to also significantly alter the expression of the genes *waaV* and *waaL* (see Fig. S1).

Before studying the ability of Δ*waaW* and Δ*ychH* mutants to adhere to and invade IEC, we confirmed that their growth and viability in cell culture medium were not affected (see Fig. S2 in the supplemental material). Neither of the two mutants generated showed any decrease in their ability to interact with IEC (Fig. 2A and B) compared to AIEC LF82 bacteria overexpressing the σ^E inhibitory complex RseAB, which had a significantly decreased abilities to adhere to and to invade IEC, with 19.6% ± 5.9% and 11.4% ± 5.0% residual adhesion and invasion, respectively.

Involvement of σ^E regulon members in the ability of LF82 to form biofilm. Martinez-Medina et al. reported that biofilm formation is a novel pathogenic feature of the AIEC pathovar (33), and we previously demonstrated that the σ^E pathway is involved in biofilm formation of AIEC strain LF82 (19), but the bacterial factor(s) involved remains unidentified. To elucidate whether σ^E regulon members *ychH* and *waaWVL* are involved in AIEC biofilm formation, we compared biofilm formation on a plastic surface by wild-type strain LF82, strain LF82 overexpressing RseA and -B, LF82-Δ*waaW*::pBAD24-*waaWVL*, and Δ*ychH* isogenic mutants. While inhibition of the σ^E pathway by using 20 g liter^{-1} arabinose led to a significant decrease in the ability of strain LF82 to form biofilms, the isogenic mutant deleted for *ychH* was not modified in its ability to form biofilm (Fig. 3A). In contrast, the LF82-Δ*waaW*::pBAD24-*waaWVL* strain had a strongly decreased biofilm formation ability in the absence of arabinose, similar to that observed with inhibition of the σ^E pathway (Fig. 3A). Of note, type 1 pilus and flagellum expression levels were unchanged in the LF82-Δ*waaW*::pBAD24-*waaWVL* strain (see Fig. S3 in the sup-

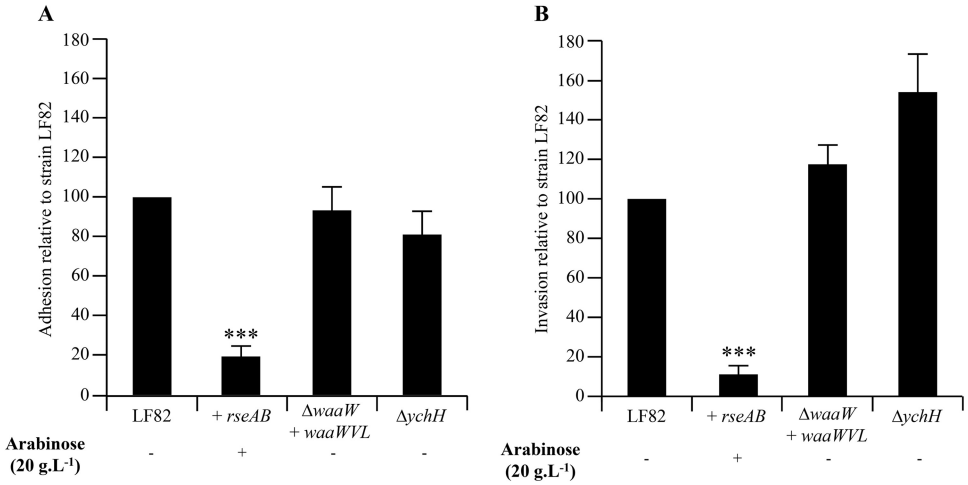

FIG 2 Involvement of the σ^E-mediated pathway in the ability of LF82 to interact with host cells. Adhesion (A) and invasion (B) abilities of LF82, LF82::pBAD24-*rseAB* (in the presence of 20 g liter^{-1} arabinose), and LF82-Δ*waaW*::pBAD24-*waaWVL* and LF82-Δ*ychH* mutants (in the absence of arabinose) with intestinal epithelial cells (I-407). Each value is the mean ± SEM of at least four separate experiments. ***, $P < 0.001$.

plemental material). The defect in biofilm formation of strain LF82-Δ*waaW*::pBAD24-*waaWVL* was fully restored in the presence of 5 g liter^{-1} arabinose, revealing that complementation of the LF82-Δ*waaW* mutant fully restored a WT-like phenotype (Fig. 3B). Overexpression of the *waaWVL* operon in nonpathogenic *E. coli* strain MG1655 was not sufficient to induce increased biofilm formation (see Fig. S4 in the supplemental material). In addition, we observed an increased level of *waaWVL* mRNA in LF82 bacteria forming biofilms, compared to planktonic bacteria grown in the same minimal medium (Fig. 3C). The induced expression of *waaWVL* during biofilm formation was parallel to that of the σ^E-encoding gene (Fig. 3C), similar to what we observed when LF82 bacteria were grown in various minimum media (see Fig. S5 in the supplemental material), supporting the idea that WaaWVL factors are the key missing elements involved in σ^E inhibition pathway-associated phenotypes.

The biofilm formation abilities of strain LF82 and mutant LF82-Δ*waaW*::pBAD24-*waaWVL* were also studied on the surface of PFA-fixed intestinal epithelial cells in the absence of arabinose. Compared to strain LF82, which was able to form a strong biofilm on the surface of the intestinal epithelial cells, as observed in the *z*-section, the mutant LF82-Δ*waaW*::pBAD24-*waaWVL* was unable to produce such a biofilm, with only a few diffusely adhering bacteria observed on the surfaces of intestinal epithelial cells (Fig. 4A). Computational analysis of these biofilm structures with the computer program COMSTAT1 (37) revealed a dramatic 91.3-fold decrease in the biofilm thickness of the LF82-Δ*waaW*::pBAD24-*waaWVL* strain compared to that of the LF82 wild-type strain ($P < 0.05$) (Fig. 4B). A 2.3-fold increase in the roughness coefficient was also observed for the LF82-Δ*waaW*::pBAD24-*waaWVL* strain compared to the LF82 strain, indicating increased biofilm heterogeneity with microcolonies (Fig. 4B).

Finally, the role of WaaW in bacterium-intestinal mucosa interactions was analyzed by using an intestinal ileal loop assay as an *in vivo* model. Intestinal ileal loops were inoculated with a mixed inoculum comprising equivalent numbers of wild-type LF82 bacteria and LF82-Δ*waaW*::pBAD24-*waaWVL* bacteria in the absence of arabinose, and the strains were compared by competitive index (CI) analysis, which provided a sensitive measurement of the relative degree of attenuation (39). The analyses of *in vitro* cocultures of LF82 wild-type bacteria and LF82-Δ*waaW*::pBAD24-*waaWVL* bacteria in the absence of arabinose revealed that both strains remained stable over time (see Fig. S6 in the supplemental material), and the intestinal ileal loop assays revealed that LF82 with inhibition of *waaWVL* expression had a mean CI of 0.38 ± 0.05, indicating that the depletion of WaaWVL expression greatly impaired intestinal mucosa colonization ($P < 0.0001$) (Fig. 4C).

***waaWVL* overexpression in a σ^E mutant restores a wild-type-like phenotype.** Several observations suggested that WaaWVL expression is the missing link between σ^E pathway activation and the ability of AIEC to form biofilms and to colonize the intestinal mucosa. First, similar phenotypes were observed between strains impaired in the σ^E pathway or in WaaWVL expression (19) (Fig. 3A). Moreover, a perfect correlation occurred between σ^E pathway activation and WaaWVL factor synthesis during the biofilm formation process (Fig. 3C). In order to test this hypothesis, we next analyzed if *waaWVL* overexpression was able to reverse phenotypes observed in a σ^E mutant to "WT-like" phenotypes. For this purpose, pBAD24-*waaWVL* was transformed in AIEC strain LF82 overexpressing the σ^E pathway inhibitors RseA and -B (the *rseAB* operon was subcloned into the pBAD33 vector in order to have antibiotic and replication origin compatibility). *waaWVL* overexpression was found to be sufficient to fully restore a WT-like phenotype in AIEC strain LF82 overexpressing the RseAB inhibitory complex, at both biofilm formation (Fig. 5A) and intestinal mucosa colonization (Fig. 5C). As previously observed, *rseAB* overexpression and/or *waaWVL* overexpression had no effect on biofilm formation or on intestinal mucosa colonization of strain MG1655 (Fig. 5B and D).

WaaWVL factors are not involved in biofilm formation abilities of all *E. coli* strains belonging to the B2 phylogroup. BLAST analysis (http://blast.ncbi.nlm.nih.gov) showed that the *waaWVL* operon is also present in two other sequenced AIEC strains (NRG 857C [48] and UM146 [49]), as well as in urinary pathogenic *E.*

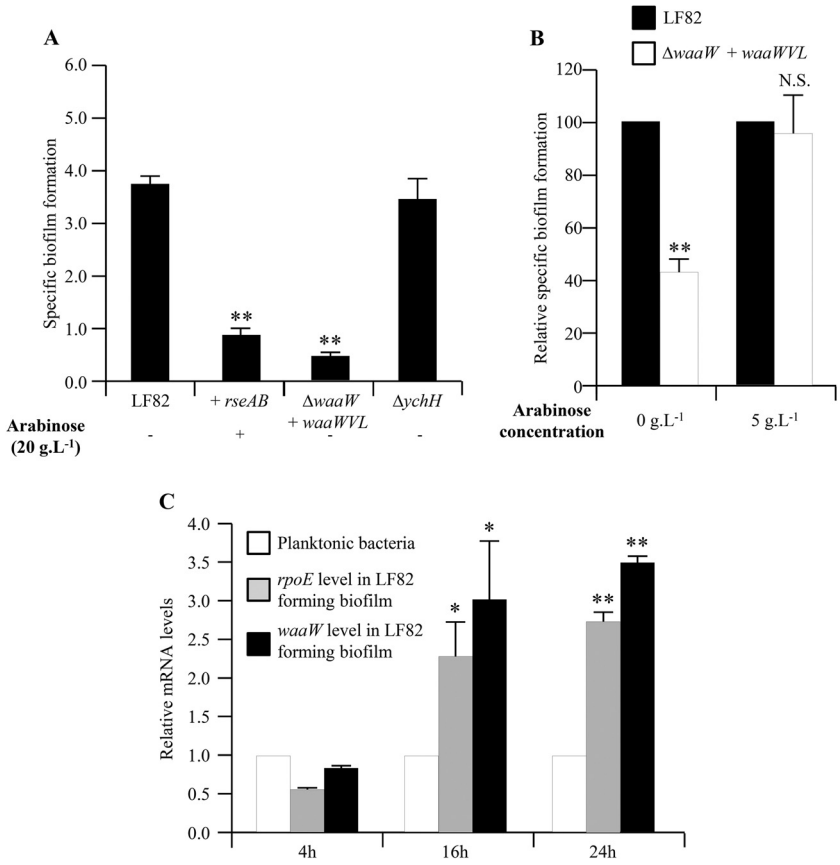

FIG 3 Involvement of the *waaWVL* operon in biofilm formation by AIEC strains. (A) SBF index of AIEC strains LF82, LF82::pBAD24-*rseAB* (in the presence of 20 g liter^{-1} arabinose), and LF82-Δ*waaW*::pBAD24-*waaWVL* and LF82-Δ*ychH* mutants (in the absence of arabinose). Data are means ± SEM from three separate experiments. (B) Biofilm formation abilities of AIEC strain LF82 and LF82-Δ*waaW*::pBAD24-*waaWVL* mutants in the absence or in the presence of 5 g liter^{-1} arabinose. Data are means ± SEM from three separate experiments. LF82 wild-type strain was defined as 100%. (C) Activation of the σ^E pathway and *waaW* expression in AIEC strain LF82 during the biofilm formation process. The fold variation of *rpoE* and *waaW* mRNA levels in wild-type strain LF82 forming biofilm (at 4 h, 16 h, and 24 h), relative to those of the wild-type strain grown in M63 broth. 16S rRNA levels were measured as a reference. Data are means ± SEM from three separate experiments. *, $P < 0.05$; **, $P < 0.01$.

coli strains CFT073 (50), UTI89 (51), and 536 (52), which are responsible for urinary tract infections, and in avian pathogenic *E. coli* (APEC) strain 01 (53) (see Fig. S7A in the supplemental material). All these strains, including AIEC LF82, belong to the phylogenetic group B2, and sequence analysis revealed that the AIEC strain LF82 genome is close to those of UPEC and APEC strains (18). Comparison of nucleotide sequences showed that *waaWVL* operons shared 99 to 100% homology between these strains (see Fig. S7B in the supplemental material), suggesting that these factors may be involved in biofilm formation in UPEC and APEC strains.

We therefore generated Δ*waaW*::pBAD24-*waaWVL* constructs in UPEC strains (CFT073, UTI89, and 536), APEC strain 01, and commensal *E. coli* strain HS. We observed that the abilities of wild-type UPEC, APEC, and commensal strains to form biofilms were significantly lower than those of AIEC strains LF82 and LF134 (Fig. 6). Growth of these strains was not affected after *waaW* depletion (see Fig. S8 in the supplemental material), and unexpectedly, none of them was affected in biofilm formation ability after WaaWVL depletion, compared to AIEC strains LF82 and LF134, for which a significant decrease in biofilm formation was observed after *waaW* depletion (Fig. 6). These data suggest that the involvement of the *waaWVL* operon in mediating biofilm formation is a mechanism specific to AIEC strains.

WaaWVL depletion leads to modification of the LPS structure in AIEC strain LF82. Based on previous report describing the involvement of WaaW, WaaV, and WaaL proteins in LPS biosynthesis (54–59), LPS from LF82 and LF82-Δ*waaW*::pBAD24-*waaWVL* strains grown in the absence or in the presence of arabinose were extracted and purified using the hot phenol-water procedure before silver staining, PAS staining, and anti-O83 immunoblotting. PAS staining and the use of an antibody directed toward the O-antigen of LPS revealed a ladder-like pattern of LPS that ranged in size from 20 to 70 kDa for all three strains used (Fig. 7A to C). In the absence of arabinose, the LF82-Δ*waaW*::pBAD24-*waaWVL* mutant exhibited differences in the LPS pattern compared to the WT AIEC strain LF82, as revealed by silver and periodic acid-Schiff staining. In particular, we noticed the accumulation of shorter semirough LPS species (around 20 and 27 kDa) after *waaWVL* depletion, indicating impaired LPS synthesis. In agreement with their identification as biosynthetic intermediates and consequently devoid of polymerized O-polysaccharides, these two bands were not revealed by the anti-O83

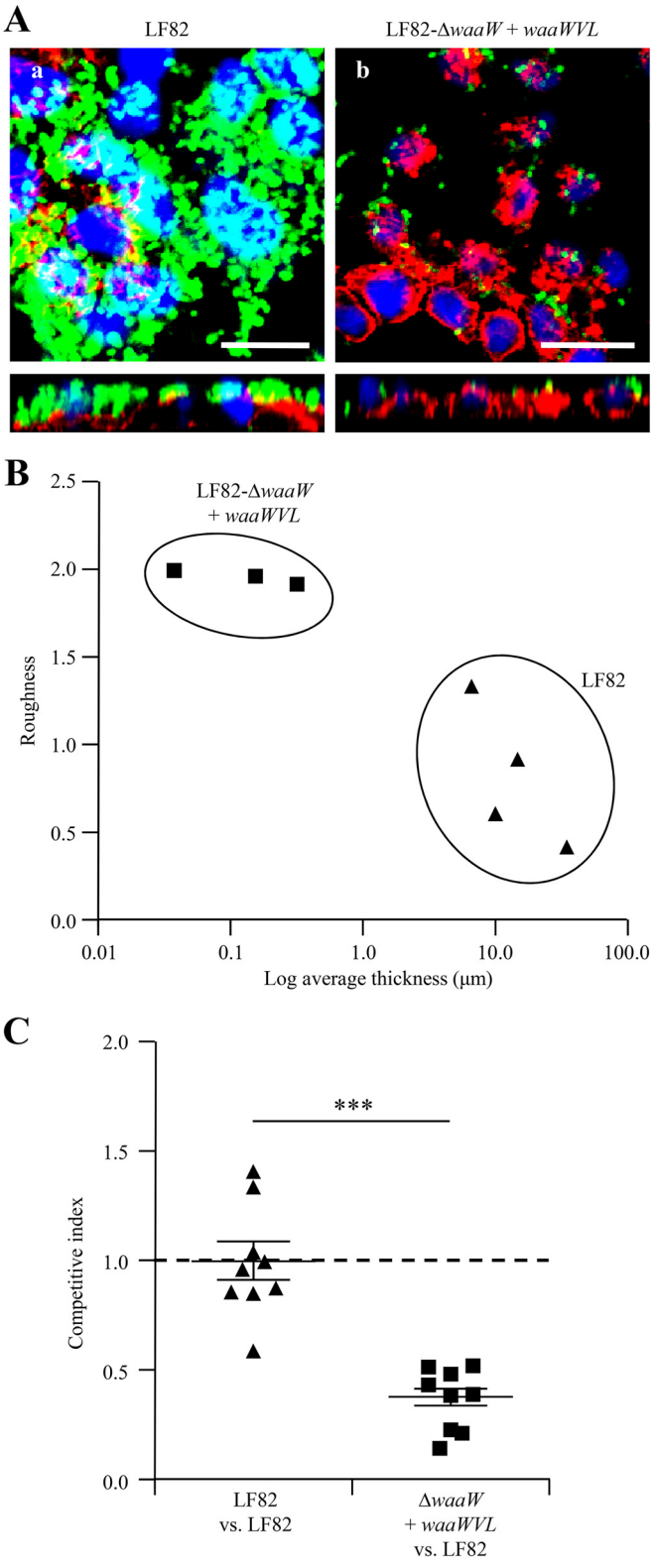

FIG 4 Involvement of the *waaWVL* operon in the AIEC strain LF82 interaction with the intestinal mucosa. (A) Confocal analysis of LF82 and LF82-Δ*waaW*::pBAD24-*waaWVL* biofilm formation at the surface of a PFA-fixed monolayer of intestinal epithelial I-407 cells in the absence of arabinose. Bacteria expressing GFP were used. Actin is labeled in red (with phalloidin-TRITC), and nuclei are labeled in blue with Hoescht. Bar, 50 μm. (B) Roughness versus average thickness of the biofilm structure of LF82 and LF82-Δ*waaW*::pBAD24-*waaWVL* on the surface of PFA-fixed monolayers of intestinal epithelial I-407 cells at 24 h in the absence of arabinose. Images were quantified using the computer program COMSTAT, and each spot represents results from an independent experiment. (C) The CI of the LF82-Δ*waaW*::pBAD24-*waaWVL* strain compared to the LF82 wild-type strain in the absence of arabinose. Intestinal ileal loops were inoculated by mixed inocula comprising equivalent numbers of wild-type LF82 and LF82-Δ*waaW*::pBAD24-*waaWVL* bacteria, and the strains were compared by CI analysis. ***, $P < 0.001$.

antibody. Importantly, a normal WT-like LPS pattern was observed when the strain LF82-Δ*waaW*::pBAD24-*waaWVL* was grown in the presence of 5 g liter^{-1} arabinose (Fig. 7A to C), revealing that complementation of the LF82-Δ*waaW* mutant fully recovered the impaired LPS synthesis. These data were subsequently confirmed in NMR experiments (Fig. 7D). Ring protons from sugar classically resonate between 3 and 5.5 ppm, and the superimposition of the three one-dimensional ^{1}H-NMR spectra of wild-type AIEC strain LF82 and the LF82-Δ*waaW*::pBAD24-*waaWVL* strain (with or without arabinose) indicated differences in the signal intensities corresponding to sugar molecules. Those domains of the spectra correspond to beta-anomeric proton spin systems, indicating that these are beta-monosaccharides (a chain of β-Glc) mainly affected by the number of glucose repetitions. NMR signals are quantitative, and we identified a loss of approximately 20% of β-Glc chain length, which can be correlated with impaired LPS biosynthesis associated with an overall shortening of the LPS population and accumulation of LPS intermediates in the LF82-Δ*waaW* mutant (Fig. 7).

DISCUSSION

Among bacteria adherent to the ileal mucosa of CD patients, adherent-invasive *E. coli* has been observed (7), and we have identified type 1 pili and flagella as important virulence factors mediating the interaction of bacteria with intestinal epithelial cells (13, 14). The role of the σ^E pathway in the ability of *E. coli* to interact with intestinal epithelial cells was previously reported for AIEC strain LF82 (19), with the observation that inhibition of this pathway greatly decreased adhesion and invasion processes (19). This involvement of the σ^E pathway in adhesion/invasion phenotypes of AIEC strains was found to be linked to expression of flagella and type 1 pili. Moreover, inhibition of the σ^E pathway led to a decreased ability of AIEC strain LF82 to form biofilms, which was previously reported to be another characteristics of AIEC strains (33), and such decreased biofilm formation was not observed with nonpathogenic *E. coli* K-12 strain MG1655 (19). These findings suggested that any gene whose transcription is under the control of σ^E could be involved in biofilm formation by AIEC strains and that this factor(s) or its σ^E-dependent expression is absent in K-12 strain MG1655.

In this study, the identification of genes whose transcription is under the control of the σ^E factor and are putatively involved in biofilm formation as well as in intestinal mucosal colonization was performed, based on a previous study by Rhodius and collaborators, which identified the σ^E regulon in *E. coli* K-12 strain MG1655 by using a defined σ^E consensus fixation domain and *in silico* genome screening (23). In the present study, we precisely defined a σ^E consensus fixation domain based on the analysis of 28 *E. coli* genes known to be σ^E regulated, and we subsequently screened the genomes of AIEC LF82 and *E. coli* K-12 MG1655 strains in order

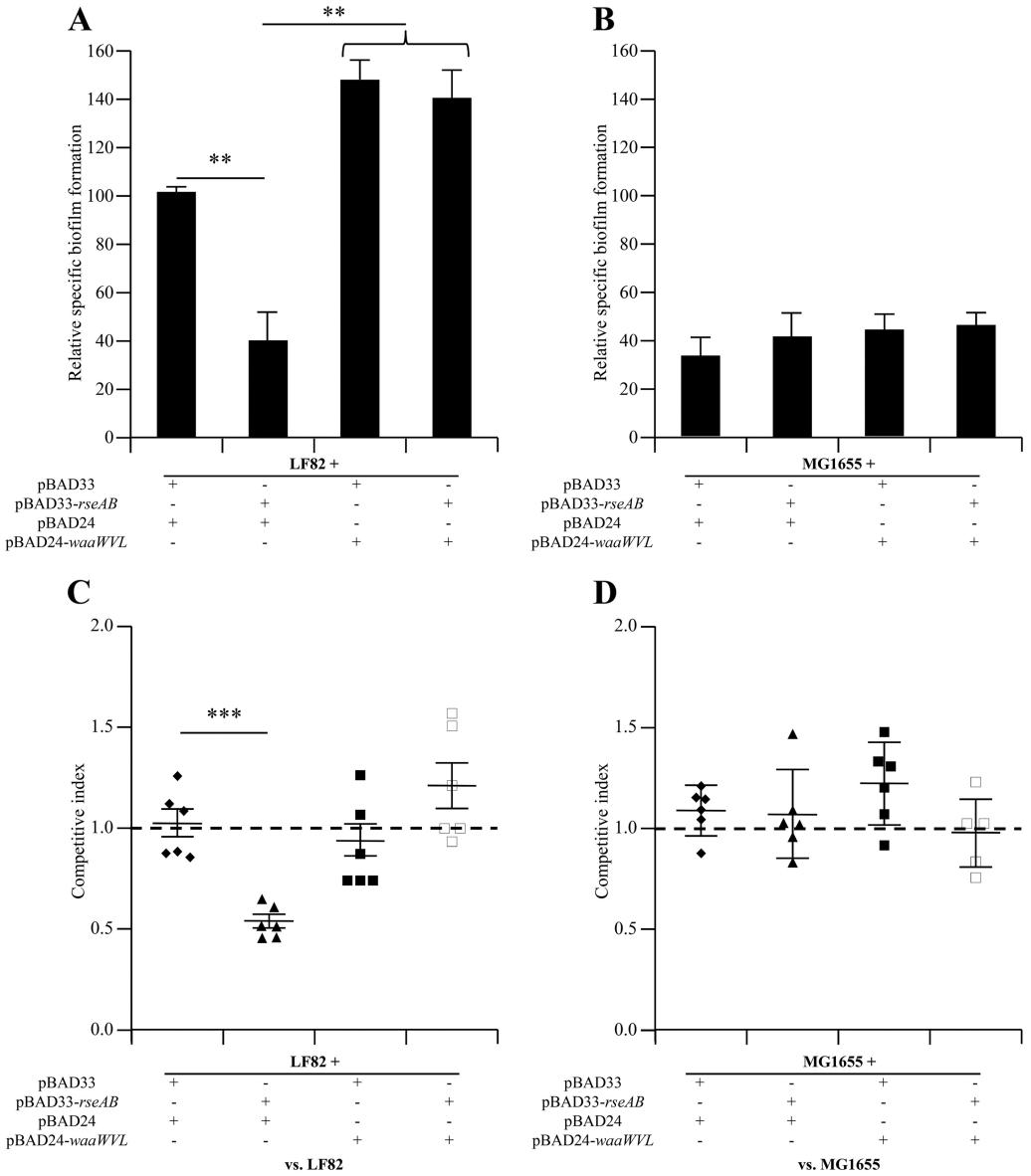

FIG 5 *waaWVL* overexpression complement σ^E mutant phenotypes in AIEC strain LF82. (A) SBF index of LF82::pBAD33::pBAD24, LF82::pBAD33-*rseAB*::pBAD24, LF82::pBAD33::pBAD24-*waaWVL*, and LF82::pBAD33-*rseAB*::pBAD24-*waaWVL* strains in the presence of 20 g liter^{-1} arabinose. Data are means ± SEM from three separate experiments. The result with the LF82 wild-type strain was defined as 100%. (B) SBF index of MG1655::pBAD33::pBAD24, MG1655::pBAD33-*rseAB*::pBAD24, MG1655::pBAD33::pBAD24-*waaWVL*, and MG1655::pBAD33-*rseAB*::pBAD24-*waaWVL* strains in the presence of 20 g liter^{-1} arabinose. Data are means ± SEM from three separate experiments. The result with the LF82 wild-type strain was defined as 100%. (C) Competitive index of LF82::pBAD33::pBAD24, LF82::pBAD33-*rseAB*::pBAD24, LF82::pBAD33::pBAD24-*waaWVL*, and LF82::pBAD33-*rseAB*::pBAD24-*waaWVL* strains compared to the LF82 wild-type strain in the presence of 20 g liter^{-1} arabinose. Intestinal ileal loops were inoculated with mixed inocula containing equivalent numbers of bacteria of the two strains, and the strains were compared based on CI analysis. (D) Competitive index of MG1655+pBAD33+pBAD24, MG1655::pBAD33-*rseAB*::pBAD24, MG1655::pBAD33::pBAD24-*waaWVL*, and MG1655::pBAD33-*rseAB*::pBAD24-*waaWVL* strains compared to results with the MG1655 wild-type strain in the presence of 20 g liter^{-1} arabinose. Intestinal ileal loops were inoculated by mixed inocula containing equivalent numbers of bacteria of the two strains, and the strains were compared based on CI analysis. **, $P < 0.01$; ***, $P < 0.001$.

to identify the σ^E regulon. This screening method led to the identification of 53 genes in strain MG1655 and 52 genes in strain LF82 whose transcription levels are putatively regulated by σ^E. Comparison of these two sets of genes allowed the identification of 12 genes specific to AIEC strain LF82. Of note, the analysis of the LF82 σ^E regulon did not identify any σ^E consensus fixation domain upstream of *fim* and *fli* operons, encoding, respectively, type 1 pili and flagella, nor upstream of intermediate factors involved in transcriptional activation of these operons, revealing that additional experiments are needed to decipher the molecular mechanism that lead to decreased expression of type 1 pili and flagella during σ^E pathway inhibition. A recent study showed that the σ^E pathway promotes flagellum expression in *Salmonella enterica* serovar Typhi under hyperosmotic stress (60), but the mechanism involved has not yet been identified.

Analysis of phenotypes associated with deletion of the 12 AIEC

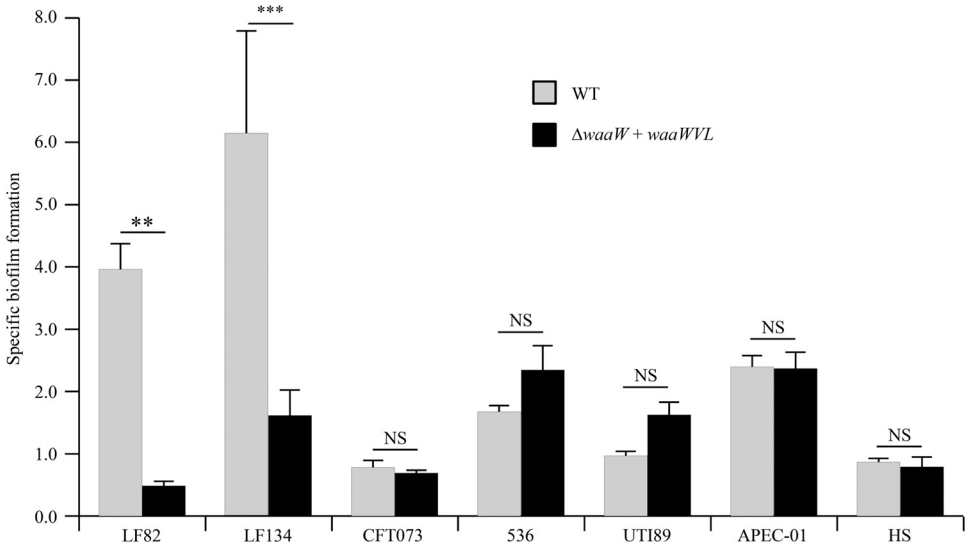

FIG 6 The *waaWVL* operon is not involved in biofilm formation of UPEC, APEC, and HS strains. SBF indexes of AIEC strains LF82 and LF134, UPEC strains CFT073, 536, and UTI89, APEC strain 01, and commensal *E. coli* strain HS and of the Δ*waaW*::pBAD24-*waaWVL* constructions in the absence of arabinose. Data are means ± SEM from four separate experiments. **, $P < 0.01$; ***, $P < 0.001$.

strain LF82-specific σ^E regulon members showed that none of them was involved in the adhesion and invasion processes, but that one of them, the *waaWVL* operon, plays a crucial role in biofilm formation. Indeed, the decrease in biofilm formation after *waaW* depletion was similar to the decrease observed after σ^E pathway inhibition. In addition, we used an *in vivo* intestinal ileal loop assay model to perform competitive index analysis, as previously used for the identification of virulence factors in *Salmonella* spp. (39) and *Listeria monocytogenes* (61), providing a sensitive measurement of the relative degree of attenuation of wild-type strains and corresponding mutants. We identified that, after depletion of the WaaW-encoding gene, the presence of AIEC LF82 bacteria on the surface of murine intestinal mucosa was dramatically impaired. Of note, flagellum and type 1 pilus expression levels were unchanged after depletion of WaaW, demonstrating that these two factors were not involved in the decrease of biofilm formation and intestinal mucosa colonization observed. The analysis of biofilm-associated AIEC LF82 bacteria revealed that both σ^E and *waaWVL* expression levels are highly activated during the biofilm formation process, suggesting that both play a key role in this process. Importantly, we observed that *waaWVL* overexpression fully restores a wild-type phenotype in AIEC strain LF82 that overexpresses the RseAB inhibitory complex, as shown based on biofilm formation and intestinal mucosa colonization levels. Furthermore, *rseAB* overexpression and/or *waaWVL* overexpression did not modify the weak ability of K-12 *E. coli* strain MG1655 to form biofilms or to colonize the intestinal mucosa.

BLAST and Interproscan analyses revealed that the three enzymes encoded by the *waaWVL* operon are WaaW, an LPS α-1,2-galactosyl transferase, WaaV, a β-1,3-glucosyltransferase, and WaaL, a lipid A-core surface polymer ligase with an O-antigen ligase-related domain. These three enzymes are predicted to be involved in LPS biosynthesis and, more specifically, in core oligosaccharide and O-antigen biosynthesis (54, 55). Multiple reports have revealed that modification of LPS composition and/or length can affect the virulence of pathogenic bacteria, as shown for *Shigella flexneri* (62), and changes in LPS composition or length can also affect biofilm formation, as reported for *Pseudomonas aeruginosa* (63) and uropathogenic *Escherichia coli* (64).

LPS analysis in AIEC strain LF82 revealed that WaaWVL depletion is associated with accumulation of shorter semirough LPS species, indicating impaired LPS synthesis. These data correlate with the previously reported functions of WaaW, WaaV, and WaaL proteins in LPS biosynthesis (54–59), and they indicate that these modifications of LPS length and structure might be the cause of the phenotypes observed with the LF82-Δ*waaW* mutant, as previously suggested for *E. coli* (65) and for *Candida albicans* (66). Based on our original finding that *waaWVL* disruption leads to lethality in various AIEC strains, but can be easily depleted without any associated lethality in nonpathogenic *E. coli* strains as well as in UPEC and APEC strains (in which WaaWVL enzymes were found to not have any effect on biofilm formation [this report and reference 58]), we hypothesize that WaaWVL enzymes may also play a role on AIEC biofilm formation in an AIEC-specific and LPS-independent manner. Such AIEC-specific involvement of WaaWVL enzymes in biofilm formation could indeed explain why *waaWVL* depletion leads to an altered biofilm formation ability only in AIEC strains, for example, by regulating AIEC-specific factor expression or membrane anchorage, resulting in an altered biofilm formation ability.

BLAST analysis indicated that the *waaWVL* operon is present in various *E. coli* strains belonging to the B2 phylogroup, including the sequenced CD-associated AIEC strains NRG857C and UM146, APEC strain 01, and UPEC strains 536, UTI89, and CFT073. Compared to the wild-type UPEC strain UTI89, a Δ*rpoE* Δ*rseAB* mutant was reported to be less able to form biofilms (67). In the present study, we demonstrated that WaaWVL factors were not involved in APEC and UPEC strains biofilm formation, since depletion of the *waaWVL* operon in APEC and UPEC strains did not lead to any modification of biofilm production. We also observed that depletion of the *waaWVL* operon did not modify the biofilm formation ability of nonpathogenic *E. coli* strain HS, as

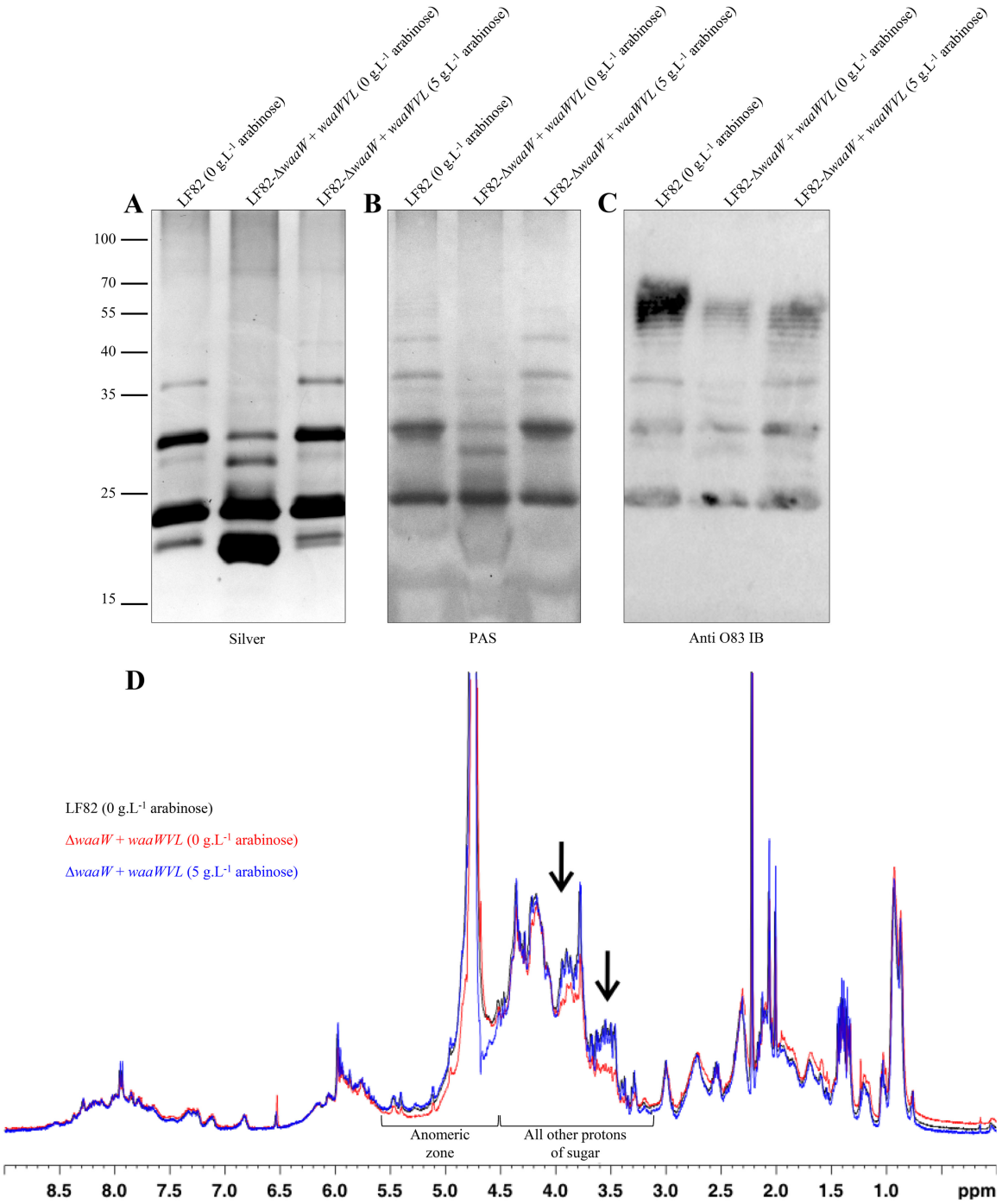

FIG 7 Analysis of purified LPS from AIEC strain LF82 and the LF82-Δ*waaW* + pBAD24-*waaWVL* mutant in the absence or presence of 5 g liter^{-1} arabinose. (A to C) Electrophoretic profile of purified LPS separated on a 15% SDS-PAGE gel and revealed by silver staining (A), periodic acid-Schiff staining (B), or anti-O83 immunoblotting (C). (D) Proton NMR spectra of LPS oligosaccharide preparations from AIEC strain LF82 and the LF82-Δ*waaW*::pBAD24-*waaWVL* mutant in the absence or presence of 5 g liter^{-1} arabinose. Arrows indicate signal differences between the various strains.

previously described for *waaL* in commensal *E. coli* (65). One explanation of such a finding is that the *waaWVL* operon might not be efficiently expressed in these strains, which would explain why they are less able to form biofilms than AIEC strain LF82. However, when additional biofilm experiments were conducted with overexpression of a cloned *waaWVL* operon in these strains, we failed to identify any increased biofilm formation (data not shown), suggesting that WaaWVL-mediated biofilm formation in AIEC strain is a specific mechanism.

As we previously reported, specific activation of the σ^E pathway occurs in AIEC strain LF82 during adhesion to intestinal epithelial cells as well as during biofilm formation processes. The

new data presented here demonstrate that the *waaWVL* operon, whose transcription (which is σ^E dependent) is activated during bacterial interaction with intestinal epithelial cells as well as during biofilm formation, plays an important role in the ability of bacteria to form biofilms and to colonize the intestinal mucosa and might play a role in AIEC colonization of the intestinal mucosa in CD patients.

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